Activation of junB by PKC and PKA signal transduction through a novel *cis*-acting element

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ABSTRACT

The product of the *junB* gene, a gene homologous to the proto-oncogene c-*jun*, is a component of transcription factor AP-1. *JunB* expression is modulated by a wide variety of extracellular stimuli, such as serum, growth factors, phorbol esters (TPA) and activators of protein kinase A (PKA). In order to study the molecular basis of this complex regulation, we have cloned the mouse *junB* gene from a genomic testis library, and characterized the *junB* promoter. Here we show that the *junB* promoter is activated by serum, TPA, and activated PKA. Sequences located between −91 and −44 are necessary for induction. These sequences contain a CAAT box, a G-C rich region and a previously undescribed inverted repeat (IR). The IR element can mediate induction by TPA and PKA when coupled to a heterologous promoter, and specifically binds a protein of 110 kD.

INTRODUCTION

The c-*jun* proto-oncogene is the cellular homolog of v-*jun*, the transforming gene of avian sarcoma virus 17 (1), and encodes a component of the TPA-inducible transcription factor AP-1 (2−5). AP-1 is composed of a group of sequence specific DNA binding proteins that binds to the TPA responsive element (TRE), thereby regulating the expression of a number of TPA-inducible genes (reviewed in refs. 6 and 7). Two other c-*jun* related genes, *junB* and *junD*, have been cloned from cDNA libraries from growth factor stimulated mouse fibroblasts (8, 9). All three Jun proteins can form heterodimers with the product of the c-*fos* gene, thereby increasing their affinity for the TRE as well as their trans-activating potential (10−15).

Both c-*jun* and *junB* are rapidly induced by agents such as growth factors and phorbol esters, whereas *junD* is relatively unresponsive (8, 9, 16−18). The *jun* genes (and c-*fos* and the fos-related genes *fosB*, *fra-1* and *fra-2* (19−21) therefore act as nuclear third messengers, converting cytoplasmic signal transduction into long term changes in gene expression. Recently it was found that the expression patterns of c-*jun* and *junB* in response to activators of the adenylate cyclase pathway as well as to membrane depolarization differ markedly (22−24). Interestingly, JunB also differs in its biological properties from c-Jun, in that it inhibits the trans-activating and transforming properties of c-Jun (25, 26). In addition, JunB is involved in the negative regulation of c-*jun* expression (25, de Groot et al., submitted). JunB is therefore likely to be a trans-repressing component of AP-1, involved in the transient nature of the nuclear response to extracellular stimuli.

Previously it was shown that the c-*jun* gene is positively autoregulated by its own gene product through binding to a TRE in its promoter (27). To determine whether the *junB* gene is also a target for regulatory action by Jun/AP1, and to study the molecular basis of the complex regulation of *junB* expression, we have cloned the murine *junB* promoter from a mouse testis genomic DNA library. In this paper we show that multiple protein-binding sequences are present in the *junB* promoter. Furthermore, we identify a new inverted repeat element (IR), which can confer TPA and PKA responsiveness to a heterologous promoter, and which binds a protein of 110 kD.

MATERIALS AND METHODS

Isolation of the *junB* promoter

A mouse balb-c testis genomic library, containing Sau3A partially digested fragments cloned into lambda EMBL-3 was screened with a mouse *junB* cDNA probe. Several *JunB* positive phages were characterized by restriction enzyme digestion, and *junB* containing DNA fragments were cloned into pGEM 3 plasmids (Promega) and were sequenced using the T7 polymerase sequencing kit (Promega).

Cells and plasmids

The mouse hepatoma cell line BW1 was a kind gift of Dr. Szpirer & Szpirer (*28*). P19 EC cells were a kind gift of Dr. McBurney (29). P19 EC and F9 EC cells were cultured in DF-Bic containing 7.5% fetal calf serum (FCS) as described earlier (30). Hep-G2 and BW1 cells were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal calf serum.

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As probes for hybridization studies, a 1.0 kb PstI mouse c-*jun* genomic fragment (de Groot et al, submitted), a 1.5 kb EcoRI cDNA fragment of *junB* (8), a 1.7 kb EcoRI cDNA fragment of *junD* (9), a 1.4 kb fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 31) and a 0.75 kb EcoRI-HindIII fragment of chicken βactin (32) were used.

The expression vectors pC-α-EV and pC-EV-X encode respectively for the wild type and mutant catalytic subunit of protein kinase A (33). Expression vectors for CREB (34), c-*jun* and *junB* (35) are described elsewhere.

JunB promoter fragments were cloned into the promoter-less CAT vector pKT. pJB1 was generated by cloning a partial *BamHI-SmaI* fragment from the *junB* 5′ regulatory region (−848−+245) into the *SmaI* site of pKT. pBJ1 contains the same fragment cloned in the reverse orientation relative to the CAT gene. pJB2 was generated from pJB1 by digestion with *XbaI* and religation. pJB3 consists of a 712 bp *SmaI* fragment cloned into the *SmaI* site of pKT. pJB4, 5 and 6 consist of a 436 bp *PstI-SmaI* fragment, a 331 bp *BglI-SmaI* fragment and a 284 bp *PvuII*(partial)-*SmaI* fragment respectively cloned in the *SmaI* site of pKT.

RNA isolation and Northern blotting

Total cellular RNA was isolated by the guanidine isothiocyanate-caesium chloride method of Chirgwin et al. (36). 15 μg of total RNA was denatured for 10 min at 68°C in 50% (v/v) formamide, 2.2 M formaldehyde, 20 mM MOPS pH 7.0, 5 mM sodium acetate, 1 mM EDTA, separated through 0.8% agarose/ 2.2 M formaldehyde gels, and subsequently transferred to nitrocellulose filters (BA 85, Schleicher & Schuell) in 20×SSC. RNA was immobilized by baking at 80°C for 2 hr under vacuum. Hybridization was performed in 50% formamide, 5× SSC, 50 mM sodium phosphate pH 6.8, 10 mM EDTA, 0.1% NaDodSO4, 0.1 mg of sonicated salmon sperm DNA per ml, 2× Denhardt solution (1× Denhardt solution contains 0.02% bovine serum albumin, 0.02% ficoll, 0.02% polyvinylpyrrolidone) at 42°C overnight. ^{32}P-labeled probes were generated using a multiprime DNA labeling kit (Amersham). After hybridization and washing, filters were exposed to Kodak XAR-5 film at −70°C using intensifying screens.

DNA transfection and transient expression assays

P19 EC and 3T3 cells were transfected as described previously (30, 37). HepG2 and BW-1 cells were plated in DMEM-Bic/7.5% FCS at 6.10^5 cells per 50 mm tissue culture dish 24 hrs prior to transfection. Two hours before transfection, the dishes received fresh medium. Cells were incubated for 16−20 hrs with calcium phosphate precipitated DNA's (10−20 μg plasmid per 50 mm dish), followed by addition of fresh medium. 16−24 hours later, the cells were harvested followed by measuring CAT activity. CAT activity was determined as described by Gorman et al. (38), and was quantitated by liquid scintillation counting of TLC-plate ^{14}C spots.

Gel shift assay and UV crosslinking

Double stranded oligonucleotides were labeled with α-^{32}PdATP and α-^{32}PdCTP (5000 Ci/mol) using Klenow fragment of DNA polymerase I. The sequence of the oligonucleotides used are : mouse c-*jun* TRE, 5′-GATCGGGTGACATCATGG-3′ (de Groot et al., submitted); mouse *junB* IR, 5′-GATCTCGG-AGTGCACTTCCG-3′. Isolation of nuclear extracts and gel shift assays were performed as described previously (35).

For UV crosslinking experiments, protein-DNA complexes were allowed to form for 20 min at 20°C. Samples were then treated with UV light (254 nm) for another 20 min at a distance of 5 cm from the light source. After this, samples were boiled in Laemmli sample buffer, and analyzed on 8−12% denaturing polyacrylamide gels.

DNase I Footprinting

DNase I footprinting reactions were performed as described by Jones et al. (39) with some modifications using nuclear extracts of P19 EC, NIH-3T3 or BW-1 cells. In short, DNA fragments were end-labeled by filling in with Klenow fragment of DNA polymerase I. Fragments were incubated in a total volume of 50 μl containing 5% v/v glycerol, 20 mM Hepes-KOH pH 7.5, 10 mM $MgCl_2$, 75 mM NaCl, 1 mM DTT, 0.18% v/v NP40 and 1 μg poly(d[I-C]) with 10−20 μg of nuclear extract. After incubation for 30−45 min. at room temperature, 4 μl of freshly prepared DNase I diluted in 50 mM $MgCl_2$ was added. Digestion was allowed for 1.5 min. at room temperature, after which 3.5 μl stop buffer (0.2 M EDTA, 2% w/v SDS) was added. Reactions were analyzed on 6% polyacrylamide/7.5M urea gels after extraction with phenol:chloroform and precipitation with isopropanol.

RESULTS

Isolation of the mouse *junB* gene

Expression of the *junB* gene is rapidly induced in response to serum, growth factors, phorbol esters and agents that raise the cytoplasmic concentration of cAMP in a wide variety of cells (8, 24, 35, 37). To study the molecular mechanisms underlying this versatile regulation, we set out to clone the *junB* gene from a mouse balb-c testis genomic library using a mouse *junB* cDNA fragment (8) as a probe. A number of positive clones were isolated, of which one (λJB31) contained the complete *junB* gene including about 8 kb of 5′ sequences (not shown). Restriction enzyme analysis and partial sequence analysis indicated that, like the human c-*jun* and *junB* genes (26, 40), the mouse *junB* gene is devoid of intervening sequences (not shown). To further study *junB* regulation, a 1100 bp *BamHI-SmaI* (−848 to + 245) fragment was subcloned and sequenced. The sequence of part of this fragment (−200 to + 100) is shown in figure 1. This fragment contains a TATA box, a CAAT box, and a GC-rich region that probably is a binding site for transcription factor zif268 (42). No TRE or SP1 binding site, both of which are present

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-200
  CCCCTGCAGCCGCGCAGAGCCACCCGGCTCGTGGCCGCTGTTTACAAGAC
-150
  ACGCGCTTCCTGACAGTGACGCGAGCCGCCTCCTCCCCTTCCCCACGCTC
-100
  TAGGAGGGGGCCGCGGGGGCCTGGCTCCGCGTCGGCCAATCGGAGTGCAC
 -50
  TTCCGCAGCTGACAAATTCAGTATAAAAGCTTGGGGCTGGGGCCGAGCAC
 +1
  TGGGGACTTTGAGGGTGGCCAGGCCAGCGTAGGATCCTGCTGGGAGCGGG
+51
  GAACTGAGGGAAGCGACGCCGAGAAAGCAGGCGTACCACGGAGGGAGAGA
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Figure 1. Nucleotide sequence of the mouse *junB* promoter. The 1100 bp *BamHI* (partial)−*SmaI* (partial) fragment (−848 to +245, see figure 2A) of the *junB* promoter was subcloned and sequenced. Only part of the sequence is shown (−200 to +100). The major transcription start site as determined by Ryder et al. (37) was assigned position +1. The TATA box is underlined, and the G-C rich region and the CAAT-box are double underlined.

in the human (40) and mouse c-*jun* promoters (de Groot et al., submitted), can be found in this fragment, nor in sequences up to −848 (not shown).

Activation of the *junB* promoter by serum, TPA and PKA

To determine whether the observed effects of serum and TPA on *junB* expression are caused by transcriptional activation of the *junB* promoter, we coupled the *BamHI-SmaI* 5′ *junB* fragment (−848 to + 245) to the bacterial chloro-amphenicol acetyl transferase (CAT) gene (Fig. 2A, pJB1) and studied its activity in transient transfection assays in a number of different cell lines. As shown in figure 2B, treatment of mouse 3T3 fibroblasts with 20% FCS causes a strong increase (8-fold) in the activity of the *junB* promoter. Similar effects were observed in human HeLa cells and mouse BW-1 hepatoma cells (not shown). In addition, TPA treatment also induces a pronounced increase in the activity of the *junB* promoter in BW-1 (Fig. 2C) and HepG2 cells (not shown). A construct containing the *junB* promoter fragment in the reverse orientation (pBJ1) was neither activated by serum (Fig. 2B) nor by TPA (Fig. 2C). Furthermore, co-transfections with the PKA expression vector p-α-EV (33) in BW-1 cells caused a strong increase in the activity of the *junB* promoter, an effect enhanced by cotransfection of a CREB expression vector (not shown). These results show that the cloned fragment contains the functional *junB* promoter, and that this promoter is activated by serum, TPA and activated PKA.

To further pinpoint the sequences responsible for the observed effects of serum, TPA and PKA on *junB* promoter activity, a number of CAT plasmids containing progressive 5′-deletions of the *junB* promoter were constructed (Fig. 2A, pJB2-6). These plasmids were transiently transfected in 3T3 and BW-1 cells, and the effects of serum, TPA and activated PKA was determined. As shown in table I, a fragment spanning *junB* sequences from −91 to +245 (pJB5) is still fully responsive to all three stimuli. Further 5′ deletion of 47 bp (pJB6, −44 to +245) however completely abolishes this inducibility, indicating that sequences between −91 and −44 are necessary for the observed effects of serum, TPA and activated PKA.

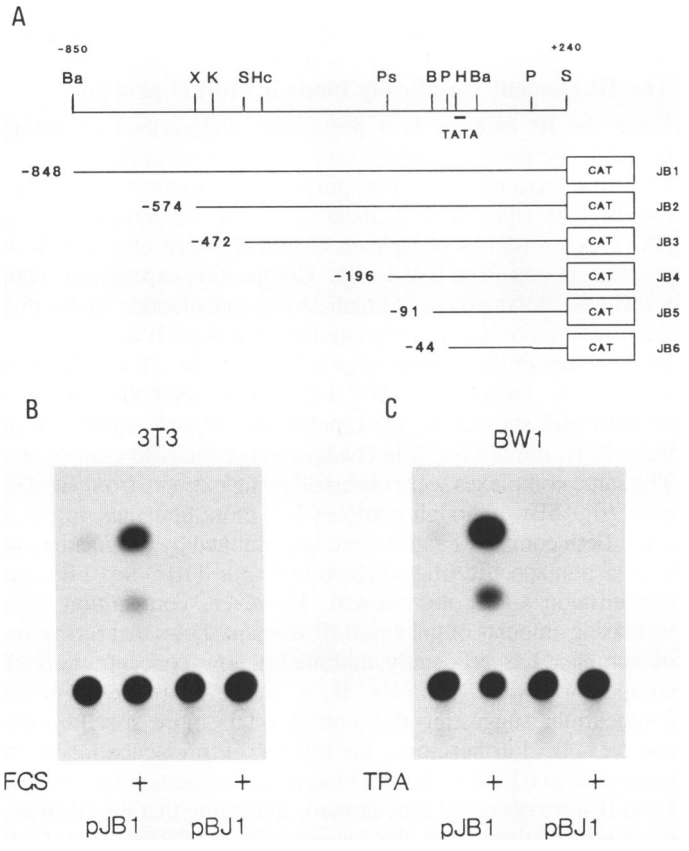

Figure 2. The *junB* promoter is activated by serum and TPA. **A.** Structure of a number of *junB* promoter-CAT fusion constructs. These constructs were made as described in the materials and methods section. Abbreviations of restriction sites are as in 1B, and K−*KpnI*, Hc−*HincII*, **B.** *BglI*, P−*PvuII*. B−Activation by serum. Mouse 3T3 fibroblasts were transiently transfected with pJB1 (see A) or pBJ1, a construct containing the *junB* promoter fused in reverse orientation to the CAT gene (10 μg per 5cm dish). 16 hours after transfection, the cells were cultured in medium containing 0.5% FCS for 8 hours, after which the dishes received fresh medium containing 0.5% FCS (−FCS) or 20% FCS (+FCS). 14 hours later, the cells were harvested and CAT activity was determined on ^{14}C TLC plates. The experiments were repeated at least 4 times, and a representative experiment is shown. **C.** Activation by TPA. Mouse BW-1 hepatoma cells, cultured in DMEM-7.5% FCS, were transfected as in B. 16 hours after transfection, the dishes received fresh medium (7.5% FCS), followed after 8 hours by addition of TPA (+TPA, 100 ng/ml) or carrier alone (−TPA) for another 14 hours. CAT activity was determined as in B.

Identification of three protein binding sites in the *junB* promoter

In an effort to define the cis-acting elements present in the *junB* promoter, we performed in vitro DNaseI footprinting experiments using nuclear extracts from both BW1 and HepG2 cells. As shown in figure 3, three protected sites in this fragment were detected. No differences between extracts from *junB*-expressing or -non-expressing cells was observed. In the region from −100 to −77, a G-C rich region is protected, which is a potential

Table I. Activity of the junB deletion constructs

Construct	Serum	TPA	PKA
JB1 (−848)	5.1	8.2	9.0
JB2 (−574)	5.9	9.0	11.2
JB3 (−472)	5.0	10.1	9.2
JB4 (−196)	6.8	9.1	9.9
JB5 (−91)	7.4	12.2	11.3
JB6 (−44)	1.1	1.2	1.6

Serum experiments were performed in 3T3 cells, and TPA and PKA experiments were performed in BW1 cells. Data are indicated as fold induction relative to un stimulated samples (serum and TPA) or samples co-transfected with mutant PKA expression vector (PKA), and are the mean of three independent experiments.

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                *         *         *       * *
Human -101 GAGGAGGGGGGCGCGGGGGCCCGGCTCCGGCGACGGCCAAT
Mouse -100 TAGGAGGGGGCCGCGGGGGCCTGGCTCCG CGTCGGCCAAT

                  *        **       * *** *       *
Human -60  CGGAGCGCACTTCCGTGGCTGACTAGCGCGGTATAAAGGC
Mouse -60  CGGAGTGCACTTCCGCAGCTGACAAATTCAGTATAAAAGC
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Figure 3. Identification of protein binding sites in the *junB* promoter. The −196 to +36 *PstI-BamHI junB* promoter fragment was end-labeled with Klenow polymerase or T4 polynucleotide kinase. The probes were incubated with BSA or nuclear extract from BW1 or HepG2 cells, and subjected to DNase I footprinting as described in the materials and methods section. Protected sites and the TATA-box are underlined. The G-C rich region is located between −96 and −79, the CAAT box between −67 and −61, and an inverted repeat (IR) between −57 and −50. Differences with the human *junB* promoter are indicated by asterisks.

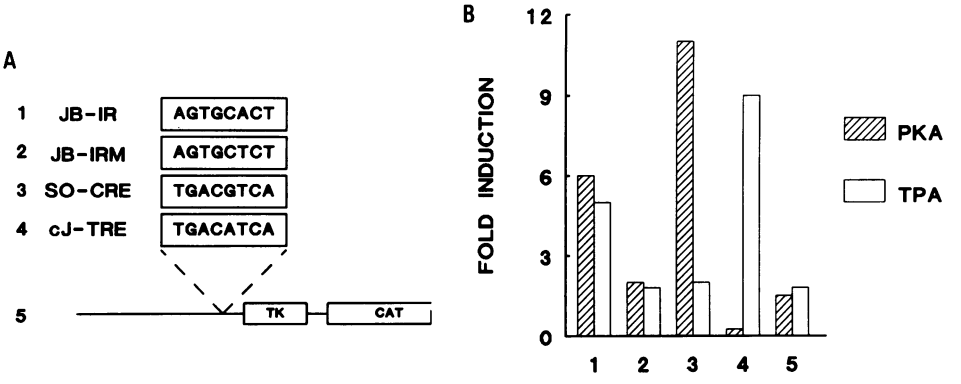

Figure 4. Identification of a PKA and PKC inducible element in the *junB* promoter. **A.** Structure of the chimeric reporter constructs containing synthetic copies of the *junB* IR (JB-IR), a point mutation of the IR (JB-IRM), the somatostatin CRE (SO-CRE) or the c-*jun* TRE (cJ-TRE) cloned in the TK-CAT vector pBLCAT2. **B.** F9 (PKA experiments) and BW-1 (TPA experiments) cells were transfected with the constructs (5 μg) shown in A or with pBLCAT2 alone (TK). PKA and CREB expression vectors (2 μg each) were co-transfected (bars marked PKA), or the cells were stimulated with TPA (100 ng/ml) for 14 hours (bars marked TPA). Total amount of DNA per dish was kept to 10 μg with pUC 18 DNA. Fold induction represents CAT activity in PKA/CREB transfected samples relative to samples co-transfected with pUC 18, or in TPA treated samples relative to untreated samples, and is the mean of three independent experiments.

binding site for zif268 (42). Further downstream, between positions −66 and −61, a typical CAAT box is protected, suggesting it is recognized by transcription factor CTF (39). A similar site is found in the human and mouse c-*jun* promoters (40, de Groot et al., submitted). The third protected region, from −57 to −50, contains a previously undescribed inverted repeat (IR; 5'-AGTGCACT-3'). All three protected regions are highly homologous to the corresponding regions in the human *junB* promoter, although only the CAAT box is identical (26). Since we found that sequences between −91 and −44 are important for *junB* induction by serum, TPA and PKA, these results suggest that the CAAT box and the IR element are important for the regulation of *junB* expression in response to these stimuli.

The IR element can mediate induction by TPA and PKC

Since induction of a CAAT box by TPA or PKA is presently unprecedented, we focussed on the IR element. To determine whether the IR element might be mediating TPA and PKA effects on the *junB* promoter, we coupled this element to a heterologous promoter (HSV-tk) and the CAT gene (JB-IR, fig. 4A), and studied its activity in transient transfection assays in F9 cells. As shown in figure 4B, the IR element from the *junB* promoter was indeed activated by PKA/CREB co-transfection, although to a somewhat lesser extent than the CRE from the somatostatin gene (SO-CRE, fig. 4A). By contrast, an A to T pointmutation in the *junB* CRE-like element (JB-IRM, fig.4A) completely inhibits trans-activation by PKA/CREB. The TRE from the c-*jun* promoter (cJ-TRE) is slightly repressed by PKA/CREB cotransfection, which is in agreement with the insensitivity of c-*jun* expression to cAMP. These results strongly suggest that the observed effects of cAMP on *junB* expression are mediated by the IR element in the *junB* promoter. Interestingly, the IR element is also significantly induced by TPA treatment, while no induction is observed with the mutated IR. The c-*jun* TRE is activated more efficiently by TPA, whereas the somatostatin CRE is not induced by TPA. Taken together, these results show that the IR element can mimic the effects of PKA and TPA on the complete *junB* promoter. By contrast, no serum induction of the IR could be detected in 3T3 cells (not shown), suggesting that additional elements in the −91 to −44 region are necessary for serum effects on the *junB* promoter.

The IR element specifically binds a 110 kD protein

Since the IR element is a previously undescribed cis-acting element, we set out to characterize the protein(s) that interacts with this sequence. For this purpose we performed gel shift experiments using the IR element as a probe. As shown in figure 5A, 2 protein-DNA complexes (I and II) were observed with nuclear extracts from BW-1 cells. Competition experiments with a 100-fold molar excess of unlabeled oligonucleotide shows that the formation of the slower migrating complex (I) is completely inhibited under these conditions, while complex II was reduced about 70%. Treatment of BW-1 cells with forskolin (fig. 5A) or TPA (not shown), which rapidly induce *junB* expression in these cells, did not result in changes in the retarded complexes. The same complexes were observed using extracts from HepG2 cells (fig. 5B), although complex I is more abundant in these cells. Both complex I and II were not inhibited by pre-incubation with a non-specific oligonucleotide (c-*jun* TRE, fig. 5B; and somatostatin CRE, not shown). However, competition with increasing amounts of unlabeled IR element shows that formation of complex I is efficiently inhibited at low concentrations of competitor, while complex II is much more resistant to competition, suggesting that complex I is more specific than complex II. Furthermore, no effects of preincubation with antibodies to CREB or Fos on binding and migration of complex I and II were observed (not shown), indicating that the IR binds protein(s) distinct from the classical PKA (CREB) and TPA (Jun/AP-1) inducible transcription factors.

To determine the molecular weight of the protein factor(s) binding to the IR, we performed UV crosslinking experiments. Nuclear extracts from BW-1 and HepG2 cells were incubated with labeled IR oligonucleotide, followed by UV treatment. Samples were then boiled in sample buffer and analyzed on a denaturing gel. With both cell types, a complex of 110 kD (complex I) and a complex of 75 kD (complex II) were observed (fig. 5C). Complex I was efficiently competed with unlabeled IR oligonucleotide (lanes S), but not with non-specific competitor DNA (lanes N). By contrast, complex II was inhibited by specific as well as non-specific competitor, indicating that complex II is probably an a-specific DNA binding protein. Similar results were observed with nuclear extracts from HeLa, P19 EC and F9 EC cells (data not shown). Taken together, these data show that a

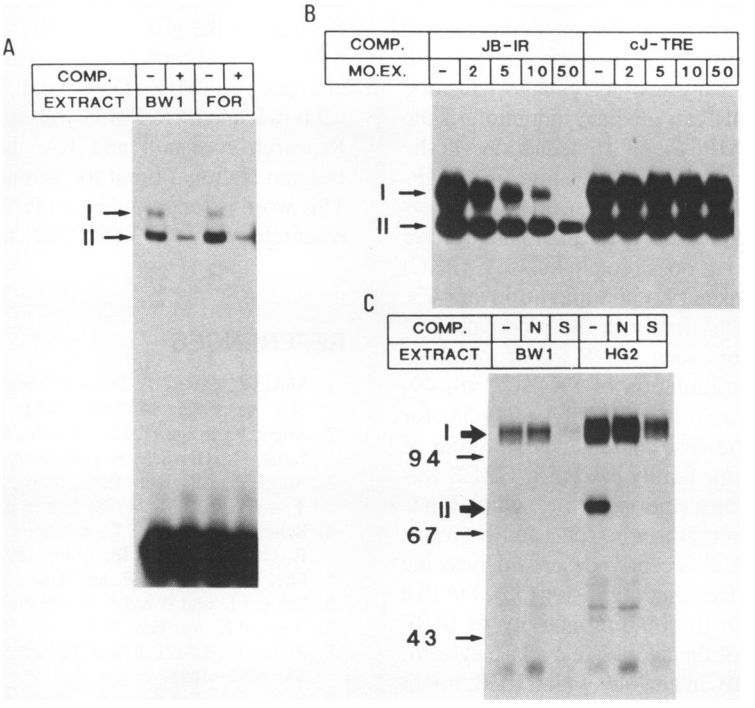

Figure 5. Specific binding of proteins to the IR element. **A.** Gel shift analysis with nuclear extracts from untreated BW1 cells (BW1) or BW1 cells treated for 1 hr with forskolin (10 μM, FOR) using an ^{32}P-labeled oligonucleotide encompassing the IR element from the *junB* promoter as a probe. Two protein-DNA complexes were detected (I & II), of which complex I was completely inhibited by pre-incubation of the nuclear extracts with a 100-fold molar excess of unlabeled IR element (+ COMP.). No differences were observed between untreated and forskolin-treated cells. **B.** Competition with homologous, but not with heterologous oligonucleotide inhibits formation of complex I. Nuclear extracts from human HepG2 cells were incubated with the labeled IR oligo alone (lanes 1 and 6), or with labeled IR oligonucleotide in combination with increasing amounts (2–50 fold molar excess) of unlabeled homologous (JB-IR) or heterologous (cJ-TRE, see figure 6A) oligonucleotide. The formation of protein-DNA complexes was monitored on a low ionic strength acrylamide gel. Only the retarded complexes are shown. Complex I is rapidly competed by unlabeled JB-IR, while complex II is only slightly competed with high amounts of this oligonucleotide. No competition of either complex was observed with unlabeled cJ-TRE. **C.** A protein of 110 kD binds specifically to the IR element. UV cross-linking experiment with nuclear extracts from BW-1 and HepG2 cells using the IR element as a probe. Protein-DNA complexes were allowed to form for 20 min. at 20°C in the absence (lanes marked -) or presence of a 25-fold molar excess of non-specific (cJ-TRE, lanes marked N) or specific (JB-IR, lanes marked S) competitor oligonucleotide, after which the reactions were treated with UV light (254 nm at a distance of 5cm) for another 20 min. Samples were then boiled in Leammli sample buffer, and analyzed on a 8% denaturing acrylamide gel. Arrows indicate the positions of the protein markers. Large arrows indicate the position of two protein-DNA complexes (I and II), and are distinct from I and II from panels A and B.

protein of 110 kD, which is expressed in a number of different cell lines, interacts specifically with the IR from the *junB* promoter. This protein will be referred to as IR binding protein (IRBP) hereafter.

DISCUSSION

The products of the c-*jun* proto-oncogene and two related genes, *junB* and *junD*, are components of transcription factor AP-1, that regulate the expression of a number of TPA-inducible genes by binding to the TRE (for a review, see refs. 6 and 7). Both c-*jun* and *junB* are rapidly induced by a wide variety of extracellular stimuli such as serum, growth factors and phorbol esters (8, 9, 16–18). In this paper we describe the isolation of the intronless mouse *junB* gene and its 5′ regulatory region. We show that the *junB* promoter is induced by serum, TPA, and activated PKA. Sequences between −91 and −44 are involved in the regulation of *junB* expression. In this region, three different protein binding sites were defined, a G-C rich region, a CAAT box and an inverted repeat (IR) element. Furthermore, we show that the IR, which specifically binds a protein of 110 kD (IRBP), can confer TPA and PKA responsiveness to a heterologous promoter.

The finding that the murine *junB* gene does not contain intervening sequences is not completely surprising, since the human c-*jun* gene is also intronless (40). However, since the DNA binding domain of all three Jun proteins is highly homologous to the DNA binding domain of the yeast transcription factor GCN4 (43), one might expect it to be located on a separate and evolutionary conserved exon. It is therefore likely that the ancestral *jun* gene, from which all three *jun* genes have probably evolved, might have lost its introns during evolution.

Using transient transfection assays, we have shown that sequences located between −91 and −44 are necessary for the induction of the *junB* promoter by serum. Although the *junB* gene is induced with similar kinetics as c-*fos* and βactin, this fragment does not contain a serum response element (SRE), which is responsible for serum induction of these early response genes (44, 45). The induction of *junB* by serum is therefore unlikely to be mediated by p67-SRF, one of the factors that bind to the SRE and is involved in serum responses (45). Footprinting analysis showed that three protein binding sites are present in the *junB* promoter, a G-C rich region (−100 to −77), a CAAT box (−66 to −61) and an inverted repeat (IR) (−57 to −50). Since only part of the G-C rich region is present in the fragment important for serum induction of *junB*, it seems not essential for this process. The CAAT box is usually recognized by transcription factor CTF (39), while the IR is a novel cis-acting element. Since CTF was not previously indicated to be involved

in serum effects on transcription, and the IR is insufficient to confer full serum inducibility to a heterologous promoter, it will be interesting to further determine the mechanism of *junB* induction by serum by molecular analysis of its regulatory factors.

Our results suggests that the IR can mediate induction of the *junB* promoter by TPA and cAMP. Signal transduction via the adenylate cyclase/cAMP system involves activation of cAMP-dependent protein kinase A (PKA). Activation of PKA relays signals generated by stimulation of the cAMP/adenylate cyclase system to the cell nucleus (41, 46) as does protein kinase C (PKC) in reaction to TPA or receptor-linked phospholipid turnover (47). Activation of these kinases results finally in the modulation of the activity of transcription factors such as NF-$\varkappa$B or members of the AP-1 gene family after stimulation of PKC (7, 48, 49) or the CREB/ATF family after activation of PKA (50–53; for review see refs. 41 and 46). The best characterized protein of the CREB/ATF transcription factor family is CREB. CREB was identified as a 43 kD nuclear transcription factor, which binds as a dimer to the CRE of the somatostatin gene and activates transcription (52, 54). Unlike the recently described tyrosine amino transferase-CRE (TAT-CRE) binding factor (55), but like the classical CREB protein (54), the binding activity of IRBP is not enhanced upon induction of the adenylate cyclase system. Transcriptional activation of IRBP, in analogy with CREB, might therefore be modulated by a PKA mediated phosphorylation event (54, 56). The binding of IRBP is furthermore only competed by the addition of a molar excess of the IR but not the somatostatin CRE (56). Moreover, despite the strong trans-activation of the IR by cotransfection of CREB, the lack of effect of a polyclonal CREB antibody on the mobility of IRBP in a gel shift assay and the discrepancy in molecular weight of both proteins argues that CREB and IRBP are distinct entities. Cotransfection of CREB might somehow modulate the activity of IRBP, thereby promiscuously activating the IR. In addition, IRBP is not likely to be a member of the Jun/AP-1 family, since: 1—the apparent molecular weight of IRBP is 110 kD; 2—pre-incubation with a Fos antibody did not result in an altered mobility of IRBP; and 3—no competition of IRBP in a gel shift was observed with TRE oligonucleotides. We therefore hypothesize that the IRBP might represent a novel transcription factor involved in nuclear signalling by both PKC and PKA. Further characterization of IRBP is currently in progress.

In contrast to the data presented here, a recent paper by Lamph et al. shows that the repression of the c-*jun* TRE by CREB is relieved when PKA is co-transfected (57). However, since these studies were performed in NIH-3T3 cells, the discrepancy with the data from F9 cells presented here might be based on cell type specific differences in proteins binding to the c-*jun* TRE.

The observed versatile regulation of *junB* is apparently necessary to correctly control the timing and magnitude of *junB* expression in response to a wide variety of extracellular stimuli. Since JunB is a trans-repressing component of AP-1 (25, 26), this complex regulation is probably of major importance for the correct regulation of TRE containing target genes by Jun/AP-1. The availability of the *junB* promoter therefore offers new possibilities for investigation of the complex genomic response to phorbol esters and other extracellular stimuli.

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REFERENCES

1. Maki, Y., Bos, T.J., Davis, C., Starbuck, M. and Vogt, P.K. (1987) Proc. Nat. Acad. Sci. **84**, 2848–2852.
2. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P. and Karin, M. (1987) Cell **49**, 729–739.
3. Angel, P., Allegretto, E.A., Okino, S.T., Hattori, K., Boyle, W.J., Hunter, T. and Karin, M. (1988) Nature **332**, 166–171.
4. Bohmann, D., Bos, T.J., Admon, T., Nishimura, A., Vogt, P.K. and Tjian, R. (1988) Science **238**, 1386–1392.
5. Lee, W., Mitchell, P. and Tjian, R. (1987) Cell **49**, 741–752.
6. Imler, J-L. and Wasylyk, B. (1989) Prog. in Growth Factor Res. **1**, 69–77.
7. Vogt, P.K. and Bos, T.J. (1989) TIBS **14**, 172–175.
8. Ryder, K., Lau, L.F. and Nathans, D. (1988) Proc.Natl.Acad.Sci. U.S.A. **85**, 1487–1491.
9. Ryder, K., Lanahan, A., Perez-Albuerne, E. and Nathans, D. (1989) Proc.Natl.Acad.Sci. U.S.A. **86**, 1500–1503.
10. Chiu, R., Boyle, W.J., Meek, J., Smeal, T., Hunter, T. and Karin, M. (1988) Cell **54**, 541–552.
11. Halazonetis, T.D., Georgopoulos, K., Greenberg, M. and Leder, P. (1988) Cell **55**, 917–924.
12. Rauscher III, F.J., Voulalas, P.J., Franza Jr, B.R. and Curran, T. (1988) Genes Dev. **2**, 1687–1699.
13. Rauscher III, F.J., Cohen, D.R., Curran, T., Bos, T.J., Vogt, P.K., Bohmann, D., Tjian, R. and Franza Jr, B.R. (1988) Science **240**, 1010–1016.
14. Sassone-Corsi, P., Lamph, W.W., Kamps, M. and Verma, I.M. (1988) Cell **54**, 553–560.
15. Smeal, T., Angel, P., Meek, J. and Karin, M. (1989) Genes & Dev. **3**, 2091–2100.
16. Lamph, W.W., Wamsley, P., Sassone-Corsi, P. and Verma, I.M. (1988) Nature **334**, 592–631.
17. Quantin, B. and Breatnach, R. (1988) Nature **334**, 538–539.
18. Ryseck, R., Hirai, S., Yaniv, M. and Bravo, R. (1988) Nature **334**, 535–537.
19. Cohen, D. and Curran, T. (1988) Mol. Cell. Biol. **8**, 2063–2069.
20. Nishina, H., Sato, H., Suzuki, T., Sato, M. and Iba, H. (1990) Proc. Natl Acad. Sci. USA **87**, 3619–3623.
21. Zerial, M., Toschi, L., Ryseck, R.P., Schuermann, M., Müller, R. and Bravo, R. (1989) EMBO J. **8**, 805–819.
22. Auwerx, J., Staels, B. and Sassone-Corsi, P. (1990) Nucl. Acids Res. **18**, 221–228.
23. Bartel, D.P., Sheng, M., Lau, L.F. and Greenberg, M.E. (1989) Genes & Dev. **3**, 304–313.
24. Mechta, F., Piette, J., Hirai, S.-I. and Yaniv, M. (1989) New Biologist **1**, 297–304.
25. Chiu, R., Angel, P. and Karin, M. (1989) Cell **59**, 979–986.
26. Schütte, J., Viallet, J., Nau, M., Segal, S.,Fedorko, J. and Minna, J. (1989) Cell **59**, 987–997.
27. Angel, P., Hattori, K., Smeal, T. and Karin, M. (1988) Cell **55**, 875–885.
28. Szpirer, C. and Spzirer, J. (1975) Differentiation **4**, 85–91.
29. McBurney, M.W., Jones-Villeneuve, E.M., Edwards, M.K.S. and Anderson, P.J. (1982) Nature **299**, 165–167.
30. De Groot, R.P., Kruyt, F.A.E., Van der Saag, P.T. and Kruijer, W. (1990) EMBO J. **9**, 1831–1837.
31. Fort, Ph., Marty, L., Piechaczyk, M., ElSabrouty, S., Dnai, Ch., Jeanteur, Ph. and Blanchard, J.M.. (1985) Nucl.Acids Res. **13**, 1431–1442.
32. Cleveland, D.W., Lopata, M.A., McDonald, R.J., Cowan, M.J., Rutter, W.J. and Kirschner, M.W. (1980) Cell **20**, 95–105.
33. Orellana, S.A. and McKnight, G.S. (1990) J.Biol.Chem. **265**, 3048–3053.
34. Gonzalez, G.A. and Montminy, M.R. (1989) Cell **59**, 675–680.
35. De Groot, R.P., Schoorlemmer, J., van Genesen, S.T. and Kruijer, W. (1990) Nucl. Acids Res. **18**, 3195–3202.
36. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry **18**, 5294–5299.

37. De Groot, R.P. and Kruijer, W. (1990) Bioch. Bioph. Res. Comm. **168**, 1074–1081.
38. Gorman, C.M., Moffat, C.P. and Howard, B.H. (1982) Mol.Cell.Biol. **2**, 1044–1051.
39. Jones, K.A., Yamamoto, K.R. and Tjian, R. (1985) Cell **42**, 559–572.
40. Hattori, K., Angel, P., Le Beau, M.M. and Karin, M. (1988) Proc. Natl. Aca. Sci. USA **85**, 9148–9152.
41. Karin, M. (1989) TIG **5**, 65–67.
42. Christy, B. and Nathans, D. (1989) Proc. Natl. Acad. Sci. USA **86**, 8737–8741.
43. Vogt, P.K., Bos, T.J. and Doolittle, R.F. (1987) Proc. Natl. Acad Sci. USA **84**, 3316–3319.
44. Treisman, R. (1986) Cell **46**, 567–574.
45. Treisman, R. (1987) EMBO J. **6**, 2711–2717.
46. Roessler, W.J., Vandenbark, G.R. and Hanson, R.W. (1988) J.Biol.Chem. **263**, 9063–9066.
47. Nishizuka, Y. (1986) Science **233**, 305–312.
48. Curran, T. and Franza, B.R. (1988) Cell **55**, 395–397.
49. Mitchell, P.J. and Tjian, R. (1989) Science **245**, 371–378.
50. Gonzalez, G., Yamamoto, K.K., Fisher, W.H., Karr, D., Menzel, P., Biggs III, W., Vale, W.W. and Montminy, M.R. (1989) Nature **337**, 749–752.
51. Hai, T., Liu, F., Coukos, W.J. and Green, M.R. (1989) Genes Dev. **3**, 2083–2090.
52. Hoeffler, J.P., Meyer, T.E., Yun, Y., Jameson, J.L. and Habener, F. (1988) Science **242**, 1430–1433.
53. Maekawa, T., Sakura, H., Kanel-ishii, C., Sudo, T., Yoshimura, T., Fujisawa, J., Yoshida, M. and Ishii, S. (1989) EMBO J. **8**, 2023–2028.
54. Yamamoto, K.K., Gonzalez, G.A., Biggs III, W.H. and Montminy, M.R. (1988) Nature **334**, 494–498.
55. Boshart, M., Weih, F., Schmidt, A., Fournier, R.E.K. and Schutz, G. (1990) Cell **61**, 905–916.
56. Montminy, M.R. and Bilezikjian, L.M. (1987) Nature **328**, 175–178.
57. Lamph, W.W., Dwarki, V.J., Ofir, R., Montminy, M. and Verma, I.M. (1990) Proc. Natl. Acad. Sci. USA **87**, 4320–4324.